

The University of Chicago

A STUDY OF THE
SUBMAXILLARY GLAND VIRUS
OF THE GUINEA PIG

A PART OF A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF BIOLOGICAL
SCIENCES IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY AND PARASITOLOGY
1935

BY

FLOYD STEPHEN MARKHAM

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A STUDY OF THE SUBMAXILLARY GLAND VIRUS OF THE GUINEA PIG *

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INTRODUCTION

Intranuclear inclusions have been described in the salivary glands of the human infant and stillborn fetus,¹ in the monkey (*Cebus fatuellus*),² guinea pig,³ rat,⁴ mouse,⁵ hamster,⁵ and mole.⁶ The presence of a filterable virus in association with these inclusions has been demonstrated only in the rodents. The benign character of these infections, and the strict host specificity and organ selectivity of these viruses set them apart as a group of particular interest. The first of the submaxillary gland viruses to be discovered was described by Cole and Kuttner⁷ in the guinea pig in 1926, and since that date the results of a number of experimental studies of this virus have appeared in the literature. The present report deals with the submaxillary virus of guinea pigs with respect to spontaneous infections, the morphology and nature of the inclusion bodies, infectivity and virulence of the virus, and the immunological phenomena associated with infection.

METHODS AND MATERIALS

Virus emulsions for experimental infections were prepared from the submaxillary glands of guinea pigs supplied by local dealers, or from artificially infected stock animals. The salivary glands were removed under ether anesthesia from 3 or more animals and ground in a mortar with sterile sand. The pulp was taken up in approximately 1 cc. of physiological saline per gland. Gross particles were allowed to settle out or were thrown down by light centrifugation. The supernatant fluid was considered as a stock emulsion for purposes of dilution. Intracerebral injections of young guinea pigs and fetuses of the same species were made in 0.05 to 0.1 cc. doses. Fetal inoculations were made by the tech-

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† Mr. and Mrs. Frank G. Logan Research Fellow.

nique described by Woolpert,⁸ employing pregnant guinea pigs in the 4th and 5th weeks of gestation. Unless otherwise specified, all tissues examined histologically were fixed in Zenker's solution, embedded in paraffin and stained with eosin-methylene blue or eosin-azure.

SPONTANEOUS INFECTIONS

The cellular changes associated with the submaxillary gland virus were first observed by Jackson in 1920.³ The inclusions are found in the epithelial cells of the salivary ducts and less often in the renal epithelium. In the submaxillary gland the affected cells undergo a marked hypertrophy, a reaction that is characteristic of many virus infections and to which Unna applied the term "ballooning degeneration." Scott and Pruett,⁹ who made a detailed study of the volumetric changes in the cell, found that under the influence of the virus the cytoplasm may increase as much as 300 per cent and the nucleus as much as 400 per cent in volume.

Lesions may occur in the mucous accessory portion of the submaxillary gland, but are most often found in the serous part where they are commonly distributed in the smaller ducts near the periphery of the lobules. A single section may disclose one or more infected ducts with or without lymphocytic infiltrations varying from a cuff three or four cells deep to massive infiltrations which completely obscure the architecture of the duct and surrounding acini. Inclusion laden cells in the latter type of lesion may stain very poorly and be difficult to find. Such reactions are observed only in old adult guinea pigs and possibly indicate a slow resolution and elimination of infected foci.

In the kidney (Fig. 5), where the epithelium of the convoluted tubules becomes infected, the affected cells do not undergo the marked hypertrophy seen in the salivary gland. The intranuclear inclusions resemble those observed in the submaxillary gland and rarely acidophilic bodies are found in the cytoplasm of affected renal epithelium.

The incidence of spontaneous infection with the submaxillary gland virus in stock animals varies widely. Andrewes¹⁰ in England reported 32 per cent. In this country Jackson found inclusions in the glands of 54 per cent, and Cole and Kuttner in 84 per cent of the animals they examined. The incidence we have found in the

stocks of local dealers ranged from 7 to 74 per cent. Casual observation suggests that there is a relation between the sanitary conditions under which the animals are kept and the frequency with which positive glands are encountered. Histological evidence of infection in the glands of guinea pigs less than 1 month old is seldom found. However, that general absence of infection in young animals is not due to temporary resistance is shown by the fact that such animals are susceptible to intracerebral¹¹ and other routes of inoculation. Spontaneous infection may take place in the first few days of life, but 10 to 15 days must elapse before typical lesions can readily be demonstrated in the glands. Of 12 14 day-old guinea pigs examined, we found microscopic evidence of infection in 1. Cole and Kuttner found typical lesions in the glands of 3 of 43 guinea pigs, most of which were less than 1 month old.

Characteristic inclusions were present in the renal epithelium of 5 of 62 of our stock animals (8 per cent). The only report in the literature mentioning the incidence of spontaneous lesions in the kidney is that of Jackson, who reported the presence of inclusions in the renal epithelium of 12 of 44 adults (27 per cent).

The natural method of transmission of the virus has not been determined under experimental conditions. The presence of inclusions in organs such as the salivary glands and kidneys suggests that their secretions may be infectious. Using Berkefeld N filtrates of urine and saliva from known infected animals, we were unable to infect young guinea pigs by intracerebral inoculation. Two weeks after injection histological examination of the brain, salivary glands and kidneys revealed no characteristic lesions.

MORPHOLOGY AND NATURE OF THE INCLUSIONS

Preliminary attempts to study the structure of the intranuclear inclusions in frozen sections of the salivary glands were abandoned because of the fragile nature of the tissues and the scarcity of isolated cells suitable for observation. It was found that the meningeal exudate of intracerebrally inoculated animals offered greater possibilities for the study of inclusions in fresh unfixed cells. Preparations for examination were made by clipping small fragments of infected meninges from the ventral surface of the brain and placing them in a small drop of serum or saline on a

slide previously coated with a solution of Janus green and neutral red. It was observed that mononuclear cells containing inclusions take up extremely small amounts of the stains as compared with uninfected cells. The former cells are further characterized by their great size and distended nuclei (Fig. 1). Lying within the nuclear membrane and sometimes attached to it by barely perceptible filaments, the inclusion appears as a closely packed mass of refractile granules. As a rule only one such mass occupies the nucleus and the surrounding nucleoplasm is optically empty. As in the fixed and stained preparations, small clumps of chromatin can be seen in apposition to the nuclear membrane.

The granular character of the inclusion as seen in fresh mounts suggested that appropriate methods might disclose corpuscles of the sort found in certain other virus inclusions. Sections 5 to 7 μ thick were prepared from Zenker-fixed submaxillary glands of spontaneously infected guinea pigs. They were treated in the usual manner with xylol, alcohol, iodine and sodium thiosulphate, but were examined in the unstained condition in glycerin mounts. In such preparations the corpuscular nature of the inclusion is strikingly apparent on direct examination, though very difficult to photograph because of the absence of contrasting stains and the oblique lighting required for their visualization (Fig. 2). They appear as very small, round or oval bodies closely packed together and embedded in a less refractile matrix or ground substance. Because of the type of illumination employed it was not possible to measure them accurately.

Various dyes were used in attempts to stain the component parts of the inclusion. The best results were obtained by over-staining with Harris' hematoxylin and differentiating in an approximately half saturated solution of picric acid. Stained by this method the corpuscles appear discrete but considerably smaller than those seen in the unstained and undehydrated glycerin mounts (Fig. 3). In a few of the inclusions so stained these minute bodies are arranged in more or less concentric rings and resemble the elementary bodies reported in infectious ectromelia inclusions photographed by darkfield illumination and ultra-violet light.¹² On the basis of the evidence presented we propose that the nuclear inclusion is composed of units comparable to the elementary bodies found in some other virus infections.

Stains for fat, fibrin and amyloid substance showed that these substances are not present in the inclusions in amounts detectable by the usual methods. Silver impregnation methods added nothing noteworthy to our information on the structure of the inclusion.

We were able to examine tissues prepared by a modification of the Altmann technique.¹³ * This method involves fixation by rapid freezing in liquid air and desiccation *in vacuo*. The dry tissues are then quickly infiltrated and embedded in paraffin and sectioned in the usual manner. The method has the great advantage of yielding tissues that are not denatured by chemical fixatives and are therefore especially suitable for microchemical studies. Sections of infected submaxillary glands were thus prepared and stained with Feulgen's reagent for thymonucleic acid. Both the cytoplasmic and intranuclear inclusions react positively but with different intensity. The cytoplasmic bodies stain rather solidly in contrast with the intranuclear inclusions which often present a marked honeycombed appearance. This seems to be due to the affinity of the matrix material alone for the staining reagent. The elementary bodies retain none of the stain, thus giving the inclusion a vacuolated appearance (Fig. 4).

Other microchemical tests were made on sections prepared by the Altmann technique. Solubility tests (test interval of 5 minutes in each instance) were made with the following reagents: petroleum ether, xylol, ethyl alcohol, physiological saline solution, 0.5 per cent acetic acid, and normal HCl at room temperature and at 60° C. None of these reagents dissolved or materially altered the appearance of the inclusions. Pepsin and HCl digested the connective tissue but not the inclusions. A 2.5 per cent solution of Na₂CO₃ removed all chromophilic matter from the inclusions and all nuclei. The Millon test for protein was positive. While none of these microchemical tests has a high degree of specificity, collectively they indicate the protein nature of the matrix-substance of the inclusion bodies.

INFECTIVITY AND VIRULENCE

Experimental transmission of the virus is readily effected in young guinea pigs by subcutaneous, intraperitoneal, or intrave-

* Through the kindness of Dr. I. Gersh, then of the Department of Anatomy, University of Chicago.

nous injections of infective gland emulsions. After 10 to 15 days, inclusions are demonstrable in the salivary glands. As in spontaneous infections, the introduction of the virus by any of these routes is not followed by any marked symptoms of disease. However, when young uninfected guinea pigs are inoculated intracerebrally, they succumb to a specific meningitis. The time of survival or death of the animal and the incubation vary with the potency of the inoculum. In those animals that die acutely the incubation period is usually 5 to 8 days. The symptoms include ruffling of the haircoat, fever, tremors, relaxation of the urethral and anal sphincters, circus movements, and finally prostration. Death is generally preceded by a marked fall in temperature.

Histological examination of the brains of guinea pigs that have succumbed to intracerebral inoculation usually reveals a marked mononuclear meningitis, characterized by the presence of large numbers of intranuclear inclusions in the cells of the exudate. When the incubation lengthens to 15 to 18 days the symptoms are less marked, and histologically the lesions are more circumscribed and the inclusions are scant.

The effect of dilution on the infectivity of the virus is shown by titration in young guinea pigs. The results of such a titration are shown in Table I.

All of the animals that received the undiluted and the 1:10 dilution of the virus emulsion died. Sections of the brains of these 4 guinea pigs showed a typical meningitis with intranuclear inclusions in the exudate. The 2 that received the 1:100 dilution responded with a definite temperature rise and minor nervous symptoms, but both survived. The 3 animals that received the 1:1000 dilution manifested no significant symptoms of infection, and histological examination of the salivary glands of 2 of these animals after 7 weeks revealed no evidence of infection. Gland emulsions, when diluted 1:100 and 1:1000 produced fatal infections in guinea pig fetuses, whereas similar dilutions of the virus produced no more than a febrile reaction in 2 weeks old guinea pigs. The fetus, therefore, proves to be a more delicate test animal for the presence of the virus. This fact is doubtless due to the generalized character of the infection in the fetus. In it lesions containing typical inclusions develop in the meninges, liver, placenta and other organs, in contrast with the localized inflammatory response in the meninges of young postnatal animals.

TABLE I
Titration of Submaxillary Gland Virus Emulsion in Young Guinea Pigs

Animal No.	Inoculum		Days after inoculation							Result
	Dilution	Dose	1	2	3	4	5	6	7	
1	Undiluted	0.10	—	+	+	+	++	++		D 7
2	Undiluted	0.05	+	+++	+++	++	—			D 6
3	1:10	0.10	—	++++	+++	+++	+++			D 5
4	1:10	0.05	—	+++	+++	+++	++	++	+	D 11
5	1:100	0.05	—	++++	+++	+++	+++	+++	+++	S
6	1:100	0.05	—	+	++	+	+++	++	+++	S
7	1:1000	0.10	—	—	—	—	—	—	—	S
8	1:1000	0.05	—	—	—	+	+	+	+	S
9	1:1000	0.05	—	—	—	+	—	+	+	S

— = 39; + = 39.0-39.5; ++ = 39.6-40.0; +++ = 40.1-40.5; ++++ = 40.6-41.0 degrees C.
D = day of death
S = survival

IMMUNITY

Although no detailed study of the immunological phenomena associated with the submaxillary gland virus has been made in the present investigation, certain facts and observations have been brought to light which have a bearing on this question.

Cole and Kuttner reported that spontaneously infected animals were refractory to intracerebral inoculation, but Kuttner¹⁴ was later unable to satisfy herself as to the presence of neutralizing antibodies in the serum of infected adults. Andrewes¹⁰ subsequently showed that if the serum from immune animals was present in the tissue culture medium, inclusion bodies were not formed in susceptible cells.

Although the guinea pig placenta is of the hemochorial type (Grosser's classification) and thus is well suited to the passage of maternal antibody to the fetus, our fetal inoculation experiments have given no evidence that there is any protection conferred upon the fetuses of an immune mother. Likewise, the young of non-immune and immune mothers have been found equally susceptible to the virus. No increased resistance to infection occurred in newborn guinea pigs which were allowed to suckle immune mothers.¹⁵ It therefore appears that passive immunization *in utero* or *postpartum* is not an important factor in resistance.

Certain observations in the fetal inoculation experiments suggest that the duration of active immunity in the spontaneously infected adult is dependent on the existence of more or less active lesions in the salivary glands or kidneys. Following intracerebral injection of the fetus, there is frequently a localization of the virus in the placenta. This oftentimes calls forth a maternal inflammatory response which breaks down the fetal epithelium and allows the liberation of infected fetal macrophages into the maternal circulation. Under such circumstances and when only a few old and well infiltrated lesions were present in the salivary glands of the mother, evidence of very recent infection of the renal epithelium was found in some of the mothers (Fig. 6), whereas signs of reinfection were not found in the kidneys of the mothers when their salivary glands contained well developed but relatively uninfiltrated foci of infection with the submaxillary gland virus.

DISCUSSION

Unlike many of the naturally occurring viruses affecting laboratory animals, the submaxillary gland virus of guinea pigs appears to be thoroughly benign and of limited distribution in tissues. In a sense it bears a certain resemblance to the so-called "opportunists" among the normal bacterial flora of the body, in that it behaves as a pathogen only when fortuitously or artificially removed from its normal habitat and introduced into very vulnerable tissues such as the meninges or those of the fetus.

In this connection it is of interest to consider the distribution of intranuclear inclusions of unknown etiology in human salivary glands and other organs. The pertinent literature was summarized by Farber and Wolbach in 1932.¹ In the review of their findings, and those of other workers, intranuclear inclusions have been reported in the duct epithelium of the salivary glands in 35 of the 51 cases studied. All 35 were infants less than 2 years of age. Of the 17 cases in which inclusions were present in other viscera, 7 were either stillborn or premature and 1 was 2 days old. Except for 1 adult, none of the 17 was more than 2 months of age.

If it be presumed that the inclusions seen in these cases are of virus etiology, it follows that nearly half the individuals with visceral lesions acquired infection *in utero*. In addition to the striking morphological similarity of the inclusion bodies in both these human and guinea pig tissues, it is to be noted that the distribution and character of the lesions in the stillborn and premature group are remarkably like those we have described in the experimental infection of the fetus with the submaxillary gland virus of the guinea pig.¹¹ Moreover, the sites of the lesions in the 35 infants between 2 months and 2 years of age is in keeping with the localization of inclusions and virus in spontaneously infected guinea pigs.

Although many investigators have suspected the virus etiology of the inclusions in human salivary glands and other organs, to date there has been no report of successful isolation of a virus by animal inoculation. If such a virus exists and has properties similar to those of the salivary gland viruses of rodents, animal inoculation as a method of isolation is not likely to succeed because the viruses of guinea pigs, rats, mice and hamsters are

strictly species specific. Therefore, tissue culture of human tissues, such as those of the umbilical cord or of the stillborn, may offer a favorable medium for the isolation of the hypothetical virus.

SUMMARY

1. The characteristics of spontaneous infection with submaxillary gland virus of guinea pigs are described. The incidence in various local stocks of guinea pigs was found to be from 7 to 74 per cent. Inclusions were found in the renal epithelial cells of 8 per cent of adult animals examined.

2. Histological evidence is presented which suggests that the inclusion bodies associated with the guinea pig salivary gland virus are composed of elementary bodies similar to those known to occur in certain other virus diseases.

3. The relative infectivity of the virus is greater for the fetus than for the postnatal guinea pig.

4. Natural passive immunization *in utero* or *per colostrum* is inadequate to protect fetuses or suckling guinea pigs against experimental infection. In spontaneously infected adults the quality or duration of active immunity may depend upon the presence of active lesions.

5. An analogy between the distribution of the inclusion bodies sometimes found in stillborn and premature human infants and in experimentally infected guinea pig fetuses is pointed out.

Note: I wish to express my appreciation to Dr. N. Paul Hudson for his advice and guidance throughout the course of this study, and to the donors of the Mr. and Mrs. Frank G. Logan Fellowship Fund.

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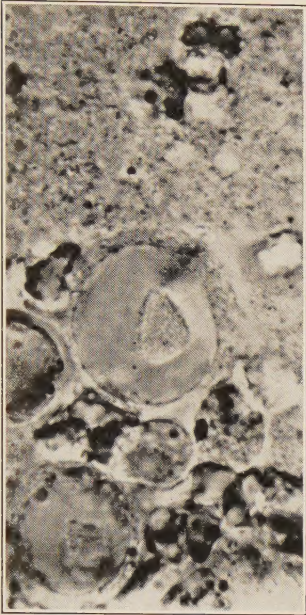
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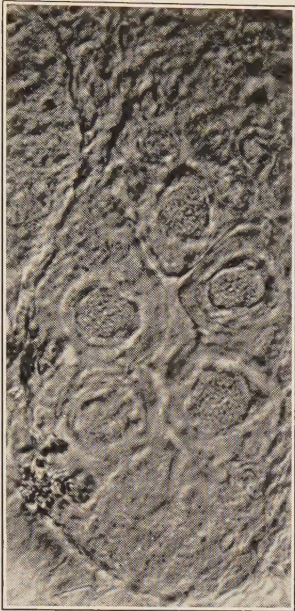
DESCRIPTION OF PLATE

PLATE 62

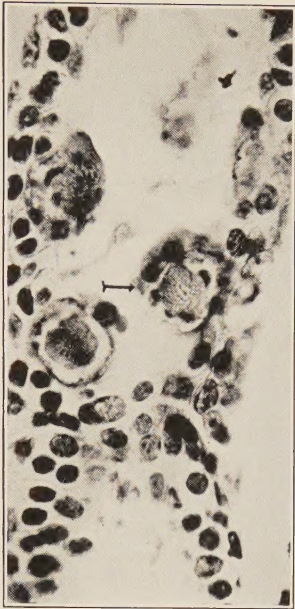
- FIG. 1. Fresh, unstained wet mount from the meningeal exudate. The intranuclear inclusion appears as a granular mass in the center of the greatly distended nucleus. $\times 1450$.
- FIG. 2. Unstained glycerin mount of Zenker-fixed submaxillary gland. The hypertrophied cells lining the duct contain intranuclear inclusions which appear to consist of corpuscles or elementary bodies of uniform size. $\times 450$.
- FIG. 3. Longitudinal section of duct in submaxillary gland stained with hematoxylin and picric acid. The arrow indicates an inclusion in which the elementary bodies are arranged in concentric rings. $\times 450$.
- FIG. 4. Feulgen stain of infected duct cells in the submaxillary gland. The cytoplasmic inclusions stain deeply while the intranuclear inclusion has a delicately honeycombed appearance and stains less intensely. Altmann fixation. $\times 1000$.
- FIG. 5. Desquamated epithelial cell in renal tubule showing an intranuclear inclusion. Hematoxylin-eosin stain. $\times 1000$.
- FIG. 6. Intranuclear inclusions in the renal epithelium of a guinea pig whose fetuses were infected *in utero*. Eosin-azure stain. $\times 1000$.



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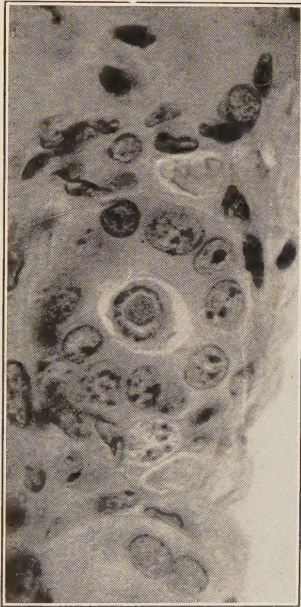
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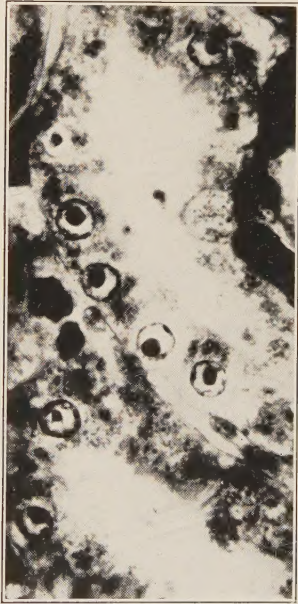
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Markham

Submaxillary Gland Virus of Guinea Pig

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